A Synopsis on

Estimation of Bacterial Diversity & Analysis of Genes Encoding Thermostable Enzymes of Thermophilic Bacteria, isolated from Arid and Semi-arid region of Rajasthan, (India)

For

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INTRODUCTION

Biodiversity is the studies on genetic, taxonomic, commercial and ecosystem aspects of living systems. Biodiversity can be defined in various ways like in microbial terms: (number of different species and their relative abundance in a given community in a given habitat), in molecular-ecological terms (number and distribution of different sequence types present in the DNA extracted from the community in the habitat). All the living individual species live in soil contain a different combination of genes and the intrinsic interaction among the gene pool influences evolution, survival and phenotypic/genotypic changes of the part of biodiversity i.e. community. Cultures isolated from the vast microbial diversity, provide some major source of biological material for the development of drugs, vaccines, pharmaceutical products, improved crops, animal varieties and for other environmental applications. The number of species of microbes in soil varies directly with environment conditions, nutrient availability, soil texture, pH, temperature, humidity, organic and inorganic nutrients like carbon and nitrogen source. The study of the microbial diversity is concern with maintenance and conservation of genetic diversity with in the population of microorganisms apart from that it is also help in to find out unknown microorganism species which can be beneficial for plant or environment.

Enzymes produced by microorganism are widely used in detergents, silver recovery, medical purposes, food processing, animal feed, pharmaceutical industries, in leather treatment and bioremediation processes. The proteases represent one of the largest groups of industrial enzymes and account for about 60 % of the world enzyme market rather the cellulose is the most abundant and renewable natural polymer on earth. Lipases occur widely in nature but only microbial lipases are commercially significant and amylases a class of industrial enzymes having approximately a 25 % share in the world enzyme market and also find potential applications in a number of industrial processes such as food (baking, brewing, dairy industries), fermentation, textile, detergent and paper industries. Microbial amylases have replaced the chemical hydrolysis of starch in starch processing industries and they would also be useful in the pharmaceutical and fine chemical industries. But the main problem with these enzymes that they denature at high temperature.

Microbial species can exist in extreme environments for example high or low temperature, high or low salt concentration and high or low pH etc. This occurrence of microbial organisms in extreme conditions is due to certain genetic and/or physiological adaptations like some alterations in the amino acid composition of proteins bring about additional electrostatic interactions, formation of hydrogen, disulfide bonds, enhancement of hydrophobic interactions or compaction of the structure and also by some proteins using the same forces involved in folding acquired by small structural modifications (Garbeva *et al.*, 2004).

Most of the thermophilic species that have been described belong to Archaea or Eubacteria. After studing and analysis this has been concluded that eubacteria are less temperature tolerant as compared to other groups. Mostly common habitats of thermophilic microorganisms are volcanically and geothermally heated hydrothermal vent systems such as

hotsprings and submarine hot vents high temperature terrestrial and marine habitats. Taxonomically thermophilic microorganisms are related to the mesophilic species. The thermophiles anatomy, ultrastructure, respiration, and metabolic processes are very similar when compared with those of mesophilic organisms. These similarities indicate that thermophiles and mesophiles have evolved from common ancestors. It also has been concluded that an enzyme from a thermophilic organism, in general, is remarkably similar to that of a mesophilic organism in physical properties (Ljungdahl and Sherod, 1960).

Thermophiles are classified into three categories according to their cardinal growth temperature like first Moderate thermophiles have adapted a very large optimum growth temperature ranging from 35 to 70° C, second Extreme thermophiles organisms have an optimum growth temperature between 55-85° C and third Hyperthermophiles can grow at very high optimum growth temperature, between 75-113° C (Baker *et al.*, 2001). On the other hand thermophiles can also be classified as Obligate thermophiles, Facultative thermophiles and Thermotolerant thermophiles. The Obligate thermophiles have optimum growth temperature between 65-75° C, do not grow below 40° C, Facultative thermophiles can grow optimally at temperature between 50-60° C, they are also able to grow at temperature around 37° C and Thermotolerant thermophiles show the maximum growth at temperature around 45-50° C sometimes they can also grow below 30° C.

Thermophiles are the organisms which are adapted to live at high temperatures. One of the most attractive attributes of thermophiles is that they produce enzymes capable of catalyzing biochemical reactions at temperatures higher than those of mesophilic organisms. The enzymes from thermophiles find a number of commercial applications because of their thermostability and thermoactivity. Themophilic microorganisms contain thermostable enzymes and these thermostable enzymes are temperature tolerant. The utility of these enzymes by thermophilic bacteria is stability of these enzymes at high temperature. Due to temperature tolerance attitude these thermophilic enzymes contain many environmental and industrial applications. Lipid, protein, cellulose and starch all molecules are present in environment in large amount therefore these thermostable enzymes have utility in biodregradation process. Proteases, cellulase, amylase and lipase enzymes also represent largest groups of industrial enzymes. Approximately 40 % of these enzymes are of microbial origin since these enzymes posses almost all characteristics desired for biotechnological applications. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new thermophilic bacteria and thermophilic industrial enzymes.

The advantage of thermophilic organisms/enzymes is that as the temperature of the process will be increase, the rate of reaction will also be increase and on the other hand these organisms/enzymes are survive/stable at high temperature which is not possible for other microorganisms therefore the higher temperature also inhibitory to microbial growth, that decreasing the possibility of microbial contamination. Cultures isolated from the vast microbial diversity provide some major source of biological material for the development of many industrial and environmental applications.

In keeping with this view that, hot Arid and Semi Arid region of Rajasthan could serve as a good source for study of microbial diversity and also new thermophilic microorganisms with novel industrially important properties. The aim of this research is the isolation and identification of bacterial species present in soil of Arid and Semi arid region of Rajasthan, further screeing of thermophilic bacteria and also estimation of industrially important extracellular enzymes producing by thermophilic bacteria. The purpose for extremophilic organisms is one of the means for obtaining enzymes with properties suitable for industrial applications. There are some advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes.

OBJECTIVES

The objectives of proposed plan are-

1. Survey and collection of soil samples.

2. Physicochemical characterization of soil samples.

3. Isolation and enumeration of total bacterial population and thermophilic bacterial strains.

4. Morphological, Cultural and Biochemical characterization of total bacteria and thermophilic strains.

5. Isolation of DNA and 16S rDNA Sequencing for genotypic characterization of total bacterial and thermophilic strains.

6. Qualitative and Quantitative estimation of extracellular enzymes of thermophiles.

7. Amplification of extracellular enzyme's gene by using specific primers.

8. Estimation of genetic diversity among all the bacterial strains.

9. Construction of dendrograms by bioinformatic tools.

REVIEW OF LITERATURE

INTERNATIONAL STATUS-

Microbial species exist in many environments that experience extremes of temperature, pH, chemical content and pressure. This occurrence is due to certain genetic and/or physiological adaptations. Stackebrandt 1994 described that the diversity provides microorganisms with a wide range of physiological and biochemical attributes. Cooney and Emerson 1964; Dix and Webester 1995; Aguilar 1996; Stetter 1999 studied on genetic survey of soil bacteria which is revealed that the primary factor that seems to govern the diversity of soil bacteria is soil pH and temperature. Torsvik and Ovreas in 2002 estimate microbial diversity and their function in soil from genes to ecosystem. Morrison *et al.*, 1924 studied on the wide distribution of thermophilic microorganisms which is shown by their existence and growth in water, sewage, snow, canned food, cotton, hey, manure, soil etc and they concluded that these microorganisms found in wide range in environment. Bergey 1919 described in their studies that true thermophilic bacteria are those which are grow at temperatures above the maximum temperature, especially the pathogenic forms, that's why according to their studies the true thermophiles show no growth or only very less growth below 40 to 45^{0}C . Aguilar 1996 studied on extremophile which are valuable resource for the exploitation of novel biotechnological process. Aguilar *et al.*, 1998 worked on a project that was able to develop integrated approach, physiological knowledge, biochemical and genetic tool needed for a better understanding and optimization of the biotechnological capabilities of extremophiles. In Scandurra *et al.*, 1998 studies described that thermostability of thermophilic microorganisms appears to be achieved by proteins using the same forces involved in folding acquired by small structural modifications in proteins. Barns *et al.*, 1996 described that with the help of recombinant DNA technology we can enhance the potential of these thermophilic organisms and also can enhance their utility. Demirjian *et al.*, 2001 concluded in their study that extremophiles and their thermostable enzymes are resistant to high temperature and show catalytic activity at high temperature. Adams and Kelly 1998 gave a review on finding and use on industrial enzymes. Ljungdahl and Sherod 1960 has been concluded that enzymes from of thermophilic organism is remarkably similar to that of a mesophilic organism in physical properties. Kristjansson 1989 described in studies that thermophilic microorganisms are denoted as sources of thermostable enzymes. Bertoldo and Antranikian 2002 studied on different mode of action of starch-hydrolyzing enzymes from thermophilic archea and bacteria. Jaeger *et al.*, 1994 studied on bacterial lipases and their importance. Lee *et al.*, 2001 in their studied discussed the production, recovery, and use of microbial lipases with the issues of enzyme kinetics, thermostability and bioactivity. Niehaus *et al.*, 1999 described the production of recombinant lipases and also discussed exteromophiles as a source of novel enzymes for industrial application. Ibrahim and El-diwany 2007 studied on thermophilic cellulases producing bacteria which have been isolated from an Egyptian hot spring

by enrichment of the water and soil samples with cellulose. Jaenicke and Böhm 1998 studied on the stability and utility of proteins in extreme environments. Somkuti and Steinberg 1986 described in their studies development of a convenient general procedure for the isolation of plasmids from thermophilic streptococci and lactobacilli that have importance in fermented dairy foods or products. Elnasser *et al.*, 2007 studied on the physiological characteristic of the wild type isolates of microbial community in the hot springs of Jordan varied over a wide range of conditions and also described their great ability to ferment different types of sugars as: glucose, fructose, manose, maltose, lactose, sucrose, trehalose, mannitol, melibiose, raffinose, xylose and cellobiose as a source of carbon, while it did not ferment xylitol and arabinose.

NATIONAL STATUS-

Bhatnagar and Bhatnagar 2005 studied on microbial types, existence and diversity in desert ecosystem. Sharma *et al.*, 2008 studied on genectic diversty of soil microbial population of western Rajasthan by RAPD analysis. Kumar *et al.*, 2000 described the protein structure that a helix-favouring residue arginine occurs more frequently whereas helix-disfavouring residues cystein, histidine and proline have lower frequencies in thermophilic proteins. Sharma and Rathore 2010 studied on lipase from twelve bacterial strains was partially purified by ammonium sulphate precipitation and dialysis method because of their medical and commercial demands and use. Ashwini *et al.*, 2011 described the optimization, production and partial purification of extracellular α-amylase from *Bacillus* sp. *Marini* in their study. Sidhu *et al.*, 1997 described the strain improvement for the production of a thermostable α-amylase in their study. Kumar and Takagi 1999 gave a viewpoint upon microbial alkaline proteases as a bio-industrial production and their utility in industries. Pandey *et al.*, 2000 gave a review on advancements in microbial amylases and their many applications/utility. Rao *et al.*, 1998 studied on molecular and biotechnological aspects of microbial proteases and also described their industrial applications. Haki and Rakshit 2003 gave a review on developments in industrially important thermostable enzymes. Sharma *et al.*, 2001 described in their study about purification and characterization of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ. Sharma *et al.*, 2001 studied on method of production, purification and characterization of lipases further they also described applications of this enzyme.

METHODOLOGY

1. **Sampling location** – Soil samples will be collected from arid and semi-arid region of shekhawati region of Rajasthan.

2. **Analysis of physico-chemical chacterization of soil** – Soil samples will be analyzed for physico-chemical characteristics include water holding capacity (WHC), humidity/moisture content, pH, electric conductivity, nitrates, phosphorous, carbonate, bicarbonate etc.

3. **Culture media and condition** – Total bacterial and thermophilic bacterial strains will be isolate on nutrient media at optimum condition. Their frequency of isolation will recorded as per standard method.

4. **Physiological/biochemical characterization** –Bacteria have a diverse set of enzymes to degrade complex compounds for energy and biomass production (Balows *et al.*, 1992). This includes studies on-
    - Temperature stress
    - pH tolerance
    - Different tests (catalase, carbohydrate utilization, IAA etc.)

5. **Enumeration of total bacteria and thermophilic bacterial population** – Total bacteria & thermophiles will enumerate by serial dilution method. Further study of thermophilic bacteria will be studied by giving them different temperatures (Holt and krieg, 1994).

6. **Genotypic characterization** – It includes-
    - Extraction and purification of genomic DNA from isolates (CTAB/Nacl method, Ausubel *et al.*, 1994).
    - PCR amplification of DNA samples will be performed by 16s rDNA (Marchesl *et al.*, 1998).
    - Sequencing of samples.

7. **Qualitative and Quantitative estimation of thermophilic bacterial extracellular enzymes**– Qualitative and Quantitative estimation of enzymes will be performed by some biochemical tests and some enzymatic experiments.

8. **Amplification of extracellular enzymes by using specific primers** - Amplification of extracellular enzymes (amylase, lipase, cellulase and protease) will be performed by using specific primers.

9. **Estimation of genetic diversity of all bacteria**– Estimation of genetic diversity among all bacterial strains will be performed.

10. **Construction of dendrograms by bioinformatic tools** – Dendrogram will be Constructed of by bioinformatic tools.

SIGNIFICANCE AND FUTURE PROSPECTS

The advantage of thermophilic organisms/enzymes is that they survive at high temperature and do stable on that condition because of that they have utility of using them at extreme condition which are not possible for other organisms. Cultures isolated from the vast microbial diversity provide some major source of biological material for the development of many industrial and environmental applications.

There are quite a few other advantages of using thermostable enzymes in industrial processes as compared to thermolabile enzymes like as in large scale fermentations with heat sensitive microorganisms, extensive efforts must be given for cooling the fermentation process but in thermophilic fermentations there is no need to be cooled. These thermophilic enzymes also contain environmental applications such as in biodegradation process of Lipid, protein, cellulose and starch molecules which are present in environment in large amount and exist in extreme environment which can not degrade by other microorganisms. On the other hand these organisms/enzymes are survive/stable at high temperature which is not possible for other microorganisms therefore the higher temperature also inhibitory to microbial growth, that decreasing the possibility of microbial contamination.

The extraordinary activity of microorganisms is based on their remarkable metabolic diversity and genetic adaptability. Rajasthan region contain extreme environment like high temperature, low rain fall, desert soil texture etc. therefore different region of Rajasthan selected and defined for bacterial strains study. As there are many environmental stress that affect microbial population present in different climatic conditions and show major diversity, this further useful in the development of technologies for the production of antibiotics and other therapeutic agents, management of pests and pathogens, bioleaching of metals, increasing soil fertility, generating biofuels, monitoring air pollution, destroying persistent pollutants, waste water treatment, bioremediation and serving tools in biomedical research. There are 90% uncultivable strains present which are useful to environment.

Future prospect includes culture of uncultivable strains of bacteria and thermophilic bacteria present in arid and semi- arid region of desert region which help in many biotechnological applications and also useful in the improvement of soil fertility

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